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Quantification of isotope-labelled and unlabelled folates in plasma, ileostomy and food samples

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ABSTRACT

New stable isotope dilution assays were developed for the simultaneous quantitation of [$^{13}C_5$]-labelled and unlabelled 5-methyltetrahydrofolic acid, 5-formyltetrahydrofolic acid, folic acid along with unlabelled tetrahydrofolic acid and 10-formylfolic acid in clinical samples deriving from human bioavailability studies, plasma, ileostomy samples and food. The methods were based on clean up by strong anion exchange followed by LC-MS/MS detection. Deuterated analogues of the folates were applied as the internal standards in the stable isotope dilution assays. Assay sensitivity was sufficient to detect all relevant folates in the respective samples as their limits of detection were below 0.62 nmol/L in plasma and below 0.73 µg/100g in food or ileostomy samples.

Quantification of the [$^{13}C_5$]-label in clinical samples offers the perspective to differentiate between folate from endogenous body pools and the administered dose when executing bioavailability trials.

INTRODUCTION

Since their discovery as a group of vitamins in 1941 [1], the dietary intake and requirements of folates are still under discussion and, despite almost seven decades of research, continuously new perceptions are attained. Basically, folates play a crucial role as coenzymes in the metabolism of one-carbon groups and their intake in Europe is considered to be below the dietary recommendations. In this context, folate deficiency is accepted to increase the risk of neural tube defects [2] and is suspected of being associated with the development of certain forms of cancer [3], Alzheimer's disease [4] and cardiovascular disease [5]. Therefore, accurate methods of folate analysis either in foods or in clinical samples are inevitable, in order to study folate metabolism and absorption kinetics.

Folate analysis is demanding due to the high number of vitamers, their occurrence in only trace amounts and their susceptibility towards light and oxygen. Stable isotope dilution assays (SIDA) using stable isotope-labelled folates and LC-MS/MS detection have proven their superiority over conventional methods in analysis of clinical and food samples [6]. For dietary recommendations, knowledge on food folate content is as essential as on folate bioavailability. However, since decades the extent of the latter is controversially discussed due to different study designs and analytical methods.

Short-term folate absorption is often assessed using plasma folate concentrations and biokinetic assays, such as the area-under-the-curve (AUC) method [7], or urinary folate excretion [8]. Another approach is to quantify non-absorbed folate in human faeces [9] or ileostomal effluent, *i.e* effluent of the small intestine, of ileostomy volunteers [10] for calculation of the absorption.

For differentiation of endogenous folate deriving from body stores and folate from a test dose, stable isotope-labelled folates have been used, on the one hand, as

tracers, and, on the other hand, as analytical internal standards (IS), with labels involving deuterium, ^{13}C or ^{15}N. The first to use folate isotopologues as tracers and as IS were Wright et al. [11], who measured [$^{13}C_6$]-labelled, [$^{15}N_{1-7}$]-labelled and unlabelled 5-methyltetrahydrofolic acid (5-CH_3-H_4folate) in the single ion monitoring mode of an LC-MS using [$^{2}H_2$]-folic acid ([$^{2}H_2$]-PteGlu) as IS. However, quantitation was hampered by spectral overlap of [$^{15}N_{1-7}$]-5-CH_3-H_4folate with, on the one hand, [$^{13}C_6$]-5-CH_3-H_4folate, and with, on the other hand, unlabelled 5-CH_3-H_4folate in single stage LC-MS. Moreover, the use of a structurally different IS, such as [$^{2}H_2$]-PteGlu, may decrease accuracy by ion suppression.A more accurate method has been reported recently by Melse-Boonstra et al. [12], who measured [$^{13}C_6$]-labelled along with [$^{13}C_{11}$]-labelled 5-CH_3-H_4folate as tracer isotopologues and simultaneously quantified unlabelled 5-CH_3-H_4folate by using [$^{13}C_5$]-5-CH_3-H_4folate as the IS. In the latter study, spectral overlaps of 5-CH_3-H_4folate isotopologues were avoided by labelling of different moiety of the target molecule and their differentiation by LC-MS/MS. However, this investigation was restricted to plasma 5-CH_3-H_4folate without an application to food or other clinical samples.

From the latter study it can be seen, that for the simultaneous use as IS and as tracer, different labels and differentiation of these isotopologues from each other and from the unlabelled folates is essential. Besides commercially available [$^{13}C_5$]-labelled folates, fourfold deuterated folates have been extensively used in folate quantitation [13]. Therefore, it appeared straightforward to use [^{13}C]-labelled isotopologues as tracers in new studies on folate bioavailability and deuterated analogues as IS for subsequent folate quantitation in clinical and food samples deriving from the studies. .

The present study was conducted to develop a method for simultaneous quantitation of these differently labelled isotopologues in clinical samples deriving from a human

ileostomy study on folate bioavailability. Ileostomy volunteers lack colon and its microflora, which might affect folate excretion. Therefore, non-absorbed folate can be estimated by quantification of post-dose folate excretion in ileostomal effluent samples. During this study, food plasma and ileostomal effluent samples were taken to determine the exact amount of [$^{13}C_5$]labelled folate in the test dose and the amount of non-absorbed folate dose, respectively. Thereafter, the amount of absorbed folate dose was calculated from the difference of the latter amounts. The assessment of folate bioavailability was based on the comparison of absorbed and non-absorbed folate.

MATERIALS AND METHODS

Materials

The following chemicals were obtained from the sources given in parentheses: Acetic acid p.a. (100 %), acetonitrile LiChrosolv, formic acid p.a. (98–100 %), n-hexan LiChrosolv, hydrochloric acid fuming 37 % LiChrosolv, methanol LiChrosolv, potassium dihydrogen phosphate, sodium acetate trihydrate p.a., disodium hydrogen phosphate p.a., sodium hydroxide p.a., water for chromatography (Merck KGaA, Darmstadt, Germany), α-Amylase, Type II-A, from Bacillus species, chicken pancreas (CP), folic acid (PteGlu), 2-(N-morpholino)ethanesulfonic acid (MES) monohydrate minimum 99.5 % titration, potassium dihydrogen phosphate dihydrate, protease Type XIV bacterial from *Streptomyces griseus*, sodiumphosphate dibasic dihydrate (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), L(+)-ascorbic acid (VWR International GmbH, Darmstadt, Germany), sodium chloride (Mallinckrodt Baker B.V., AA Deventer, The Netherlands), β-mercaptoethanol (MCE) molecular biology grade (AppliChem GmbH, Darmstadt, Germany), rat serum azide free

(BIOZOL Diagnostica Vertrieb GmbH, Eching, Germany), H_4folate, 5-CH_3-H_4folate, 10-HCO-PteGlu, 5-HCO-H_4folate (Schircks, Jona, Switzerland), [$^{13}C_5$]-5-CH_3-H_4folate, [$^{13}C_5$]-5-HCO-H_4folate, [$^{13}C_5$]-PteGlu (Merck Eprova AG, Schaffhausen, Switzerland), [$^2H_4$]-H_4folate, [$^2H_4$]-5-CH_3-H_4folate, [$^2H_4$]-10-HCO-PteGlu, [$^2H_4$]-5-HCO-H_4folate, [$^2H_4$]-PteGlu [14].

Solutions and standards

Phosphate buffer consisted of aqueous Na_2HPO_4*$2H_2O$ (0.1 mol/L) adjusted to pH 7.4 by 0.1 mol/L KH_2PO_4 solution.

MES buffer (Extraction buffer for plasma, ileostomal effluent and food samples) contained 0.02 mol/L MES monohydrate, 2% ascorbic acid and 1.4% MCE at pH 5.0.

Equilibration buffer (for SPE of plasma samples) consisted of aqueous KH_2PO_4 (3 mmol/L) adjusted to pH 7.0 by aqueous Na_2HPO_4 (6 mmol/L) and contained 0.2% MCE.

Equilibration buffer (for SPE of ileostomal effluent and food samples) contained 3 mmol/L KH_2PO_4, 6 mmol/L Na_2HPO_4 and 0.2% MCE at pH 7.5.

0.1 mol/L Phosphate buffer (0.2 mol/L MCE) was prepared by adjusting an aqueous Na_2HPO_4*$2H_2O$ solution (0.1 mol/L) to pH 7.0 with a 0.1 mol/L KH_2PO_4 solution followed by addition of 0.2 mol/L MCE. The buffers described above were prepared on day of use.

Chicken pancreas (CP) was solved in phosphate buffer (0.1 mol/L, containing 1% ascorbic acid, pH 7.0) at a concentration of 0.2 mg/mL and stirred for 15 minutes. The solution was kept at -20 °C until day of use.

Elution buffer (for SPE of plasma, ileostomal effluent and food samples) contained 5% sodium chloride, 1% ascorbic acid, 0.1 mol/L sodium acetate trihydrate and 0.1% MCE. The buffer was kept chilled and used for maximum 4 weeks.

[$^2H_4$]-labelled folate vitamers (H_4folate, 5-CH_3-H_4folate, 10-HCO-PteGlu, 5-HCO-H_4folate, PteGlu) were used as IS.

IS stock solutions contained 40 – 350 µg [$^2H_4$]-labelled folate/mL MES buffer.

IS working solutions were prepared by dilution of the stock solutions with MES buffer. IS working solutions for plasma quantitation contained 25 – 450 ng [$^2H_4$]-labelled folate/mL MES buffer, standards for ileostomy effluent and food quantitations contained 250 – 2000 ng [$^2H_4$]-labelled folate/mL MES buffer. All IS solutions were stored in amber glass bottles at -20 °C.

To determine the exact concentrations of the used IS solutions a response mixture consisting of IS solution and a solution of the corresponding unlabelled folate derivative in 0.1 mol/L phosphate buffer (0.2 mol/L MCE) or HCl (0.1 mol/L) for 10-HCO-PteGlu was analysed with every batch of samples. Concentrations of solutions of unlabelled folates were determined by spectrophotometry using a SPECORD 50 (analytikjena, Jena, Germany; H_4folate: λ = 299 nm, ε = 27.71 L/(mmol*cm); 5-CH_3-H_4folate: λ = 290 nm, ε = 23.71 L/(mmol*cm), 10-HCO-PteGlu: λ = 252 nm, ε = 24.87 L/(mmol*cm), 5-HCO-H_4folate: λ = 288 nm, ε = 23.41 L/(mmol*cm), PteGlu: λ = 282 nm, ε = 27.61 L/(mmol*cm)) prior to preparation of the response mixture. Concentration of IS working solution was subsequently determined by LC-MS/MS analysis of the response mixture considering the corresponding response curve (see below) and the amount of unlabelled folate in the mixture in a manner similar to that for determination of sample folate concentration.

Clinical samples

Plasma and ileostomal effluent samples were obtained from volunteers from a bioavailability trial [15]. The study protocol was approved by the Regional Ethical Review Board in Uppsala, Sweden.

Plasma samples derived from blood samples collected in EDTA tubes (BD Vacutainer™, Belliver Industrial Estate, Plymouth, UK) and were stored at -20 ºC until analysis.

Pooled ileostomal effluent samples were stored at -20º C until analysis.

Food samples

Wholemeal bread (8% fibre) fortified with 441 ± 54 µg (6S)-$^{13}C_5$-5-CH_3-H_4folate/100 g was baked at the Lantmännen test bakery (Järna, Sweden) as previously described [15] immediately frozen and stored at -20ºC. Folate content in the bread was quantified before and after the trial.

Precipitation of plasma protein

To prevent clotting of plasma protein on the HPLC column, precipitation and removal of plasma protein had to be accomplished during extraction. Either acetonitrile, methanol or 0.1% formic acid in these solvents are considered good precipitation reagents.

It was suspected that folates were not stable in organic solvents, as a previous report showed 12% acetonitrile to be the upper limit to prevent folate degradation [16]. Therefore, folate stability in methanol was investigated in different buffers containing 12% acetonitrile, 1% ascorbic acid, 0.1% MCE and 10%, 25% and 50% methanol, respectively, on day 1 and day 14 after preparation. To stabilize folates during protein precipitation and to avoid an additional step in the procedure, the extraction buffer

composition was modified based on the results of investigations on folate stability in acetonitrile and methanol. Modification of elution buffer composition to precipitate plasma protein on the SPE cartridge was considered another straightforward attempt to remove plasma proteins. In the course of testing seven different reagents for protein precipitation, plasma samples (n=2, 400 µl) were spiked with 100 µl 10-HCO-PteGlu (0.8 nmol/L) as IS (c.f. Table 1). 10-HCO-PteGlu was chosen as IS as it had not been detected in plasma extracted with this method previously [17].

Final extraction procedure of plasma samples

Prior to analysis, plasma was thawed at room temperature in the dark. 200 µL plasma, IS solutions ([$^2H_4$]-H_4folate, [$^2H_4$]-5-CH_3-H_4folate, [$^2H_4$]-5-HCO-H_4folate, [$^2H_4$]-PteGlu) in amounts commensurate to expected sample folate concentration (aimed peak area ratio of 0.5 - 5 for standard and analyte) and 200 µL extraction buffer were pipetted into a 2 mL Eppendorf plastic tube. A little magnetic stirrer was added, the tube was closed and samples were stirred for 37 minutes at room temperature in the dark to equilibrate analytes and internal standards. Subsequently the magnetic stirrer was removed and 600 µl methanol were added into the Eppendorf tube to precipitate plasma proteins. The tubes were closed, shaken well and centrifuged for 5 minutes at 9000 G (Centrifuge 5417C, Eppendorf, Hamburg, Germany). Supernatants were cleaned up by SPE as detailed below.

Extraction of ileostomy samples

Frozen ileostomy samples (approx. 12 g) were lyophilised in four steps of 24 h each (400 µbar -20°C, 400 µbar -10°C, 400 µbar 5°C, 0 µbar 20°C; in a model P8K-E-54-5

lyophilizer, Dieter Piatkowski, Forschungsgeräte, München, Germany) and thereafter stored at -20 ºC. For extraction, 0.5 g lyophilisate was weighted into Pyrex bottles. IS solutions ([$^2H_4$]-H_4folate, [$^2H_4$]-5-CH_3-H_4folate, [$^2H_4$]-10-HCO-PteGlu, [$^2H_4$]-5-HCO-H_4folate, [$^2H_4$]-PteGlu) in amounts commensurate to expected sample folate concentration (aimed peak area ratio of 0.5 - 3 for standard and analyte), 2 mg protease, 10 mL MES buffer, and a magnetic stirrer were added. After stirring for 5 minutes, samples were incubated in a shaking waterbath at 37°C for 4 hours. Enzyme inactivation was conducted by boiling at 100°C for 10 minutes. Samples were cooled on ice immediately. 2 mL of CP solution and 150 µL of rat serum were added prior to overnight incubation (14 h) in a shaking waterbath at 37°C. Enzymes were inactivated by boiling at 100°C for 10 minutes, before the samples were cooled on ice and centrifuged with 4000 rpm at 2°C for 25 minutes (Centrifuge CR 4-12, Jouan S.A., St. Herblain, France). Supernatants were cleaned up by SPE as detailed below.

Extraction of food samples

For extraction, 0.1 g of the sample was weighted into Pyrex bottles. IS solutions ([$^2H_4$]-H_4folate, [$^2H_4$]-5-CH_3-H_4folate, [$^2H_4$]-10-HCO-PteGlu, [$^2H_4$]-5-HCO-H_4folate, [$^2H_4$]-PteGlu) in amounts commensurate to expected sample folate concentration (aimed peak area ratio of 0.5 - 3 for standard and analyte), 3 mg α-amylase, 10 mL MES buffer, and a magnetic stirrer were added. After stirring for 5 minutes, samples were incubated in a shaking waterbath at 37°C for 2 hours. Subsequently, 2 mg protease were added and extraction and SPE clean up were conducted as described for ileostomy samples.

SPE sample clean up

Plasma samples were purified on a 12-port vacuum manifold (Visiprep, Supelco, Sigma Aldrich, Steinheim, Germany) equipped with strong anion exchange (SAX) SPE tubes (Discovery® DSC-SAX SPE tube 100 mg 1 mL, Supelco, PA, USA; Strata SPE tube 100 mg 1 mL, Phenomenex, Aschaffenburg, Germany), For extracts of ileostomal effluent and foods, the manifold was equipped with SAX SPE tubes (Discovery® DSC-SAX SPE tube 500 mg 3 mL, Supelco, PA, USA; Strata SPE tube 500 mg, 3 mL, Phenomenex, Aschaffenburg, Germany). Columns were activated by 2 cartridge volumes of n-hexane, 2 volumes of methanol, and 2 volumes of the corresponding equilibration buffer. Following sample application columns were washed with 2 (plasma) or 5 (ileostomal effluent, food) cartridge volumes of the corresponding equilibration buffer and run dry. Plasma folates were eluted with 0.5 mL elution buffer.

Two attempts were tested in order to increase assay sensitivity for ileostomy and food samples: (1) reduction of elution buffer volume from 2.0 mL to 1.5 mL and (2) fractionated elution of 1 + 1 mL. Based on these results, ileostomy and food folates were eluted with 1.5 ml elution buffer.

Liquid chromatography

Chromatography was performed on a High Performance Liquid Chromatograph (Shimadzu Corporation, Kyoto, Japan). The aqueous part of the mobile phase consisted of 1.0% acetic acid in MQ-water (A), the organic part of 0.1% formic acid in acetonitrile (B). The column effluent was directed to waste for the first seven minutes

and after 20 minutes (plasma)/ 30 minutes (ileostomal effluent/ food) during each run to avoid accumulation of salt and other interfering compounds in the MS. Injection volume was 20 µl.

For HPLC separation of plasma folates, a Nucleosil C_{18} column (150 x 2.00 mm, 3 µ, 100 Å) equipped with a C_{18} precolumn (Phenomenex, Aschaffenburg, Germany) was used. Analysis was carried out at a constant flow rate of 0.2 mL/min. Gradient elution was achieved by variation of the percentage of eluent B at the following times: 0 min 0% B, 2 min 10% B, 17 min 30% B, 19 min 100% B, 22 min 0% B, 23 min 0% B.

For HPLC separation of ileostomy and food folates, a Nucleosil C_{18} column (250 x 3.00 mm, 5 µ, 100 Å) equipped with a C_{18} precolumn (Macherey-Nagel, Düren, Germany) was used. Analysis was carried out at a constant flow rate of 0.3 mL/min. Gradient elution was achieved by variation of the percentage of eluent B at the following times: 0 min 0% B, 2 min 10% B, 25 min 25% B, 27 min 100% B, 30 min 100% B, 32 min 0% B.

To allow for column equilibration between the runs, a 15 minutes equilibration interval at 100% A was included.

Mass spectrometry

Mass spectrometry was conducted by using an API 4000 Q-Trap LC-MS/MS system (Applied Biosystems, MDS SCIEX, CA, USA) equipped with a turbo spray ion source and operated in positive ion mode. General operating conditions were chosen as follows: source temperature: 400.0°C, spray voltage: 5500.0 V, collision gas pressure: high, curtain gas: 15 psi, ion source gas 1: 38.0 psi, ion source gas 2: 30.0 psi, scan type: MRM. Analyte specific operating conditions were tuned as follows: H_4folate, [$^2H_4$]-H_4folate, PteGlu, [$^2H_4$]-PteGlu, [$^{13}C_5$]-PteGlu: DP = 53 V, EP = 8 V, CE

= 32 V, CXP = 7 V; 5-CH_3-H_4folate, [$^2H_4$]-5-CH_3-H_4folate, [$^{13}C_5$]-5-CH_3-H_4folate: DP = 71 V, EP = 4 V, CE = 23 V, CXP = 8 V; 10-HCO-PteGlu, [$^2H_4$]-10-HCO-PteGlu: DP = 59 V, EP = 9 V, CE = 38 V, CXP = 6 V, 5-HCO-H_4folate, [$^2H_4$]-5-HCO-H_4folate, [$^{13}C_5$]-5-HCO-H_4folate: DP = 54 V, EP = 6 V, CE = 23 V, CXP = 10 V. MS detection was based on the following transitions given in table 1.

Calibration (Linearity range of response)

Separate response curves were measured for each investigated folate derivative (H_4folate, 5-CH_3-H_4folate, [$^{13}C_5$]-5-CH_3-H_4folate, 10-HCO-PteGlu, 5-HCO-H_4folate, [$^{13}C_5$]-5-HCO-H_4folate, PteGlu and [$^{13}C_5$]-PteGlu) in plasma, ileostomal effluent, and foods to convert area ratios (A(IS)/A(Analyte) into molar ratios (n(IS)/n(Analyte)) to the respective internal standard ([$^2H_4$]-H_4folate, [$^2H_4$]-5-CH_3-H_4folate, [$^2H_4$]-10-HCO-PteGlu, [$^2H_4$]-5-HCO-H_4folate, [$^2H_4$]-PteGlu). IS and analyte solutions were mixed to different ratios (0.05 to 50, n=6). These mixtures were analyzed by LC-MS/MS. Response curves were determined by plotting the molar ratios against the peak area ratios and application of simple linear regression. Response stability was checked during every LC-MS/MS analysis by running a response sample of a random molar ratio.

Precision (Inter- and Intraassay-CV)

For investigation of repeatability of plasma, ileostomy and food analysis, intra-assay CV was determined by extraction of one plasma and one bread sample in triplicate according to the corresponding methods.

For determination of reproducibility of plasma, ileostomy and food assays, inter-assay CV was determined by extraction of one plasma and one bread sample in triplicate (plasma)/ duplicate (ileostomal effluent/ food) once a week for four weeks each according to the corresponding methods.

Limit of Detection (LOD)

LOD for plasma, ileostomal effluent, and food analysis were determined by extraction of sample matrices free from folates. The surrogate plasma matrix consisted of 7% lyophilised egg white and 0.06% sunflower oil in 0.9% NaCl solution. As a surrogate matrix for food and ileostomal effluent, 100% starch was used. LC-MS/MS analysis confirmed that the surrogate matrices did not contain any folates. For determination of LODs and LOQs the matrices were spiked (each in triplicate) with the analytes at four different concentration levels starting slightly above the LOD and covering 2 orders of concentration magnitude. After addition of the respective labelled internal standards, all samples underwent sample preparation and clean-up as described above and were finally analyzed by LC-MS/MS. LODs and LOQs were derived statistically from the data according to a published method [18].

Recovery and Accuracy

Matrices devoid of folates (see above) for plasma, ileostomal effluent, and food analysis were spiked with known amounts of all investigated folate derivatives (ranging between 2 to 5 nmol/L (plasma) and 0.6 to 2.5 µg/100g (ileostomal effluent and food, n=3) and subsequently extracted according to the corresponding methods.

Data analysis

All data analysis was performed using Analyst software (Applied Biosystems/ MDS, Analytical Technologies, Concord, Ontario, Canada), versions 1.4 and 1.5, from raw mass spectral data.

RESULTS AND DISCUSSION

For the application of differently labelled stable isotopologues in physiological studies, several prerequisites have to be fulfilled: a) the complete equilibration between IS and analyte(s), b) the stability of the label(s), and c) an unequivocal spectral differentiation (for details see the recent review on SIDAs for mycotoxins [19]). For the anticipated, simultaneous use of tracer isotopologues and IS isotopologues, the compliance with the former two prerequisites has been confirmed in previous method validation studies [6, 13]. However, the latter prerequisite of absent spectral overlaps had to be verified for the [$^{13}C_5$]-, the [$^{2}H_4$]-labelled and the unlabelled isotopologues of H_4folate, 5-CH_3-H_4folate, 10-HCO-PteGlu, 5-HCO-H_4folate, and PteGlu. Fig. and 2 present as an example the structures and MS/MS transitions of 5-CH_3-H_4folate isotopologues, respectively . None of the abundant product ions of [$^{13}C_5$]-5-CH_3-H_4folate was identical with those of the IS [$^{2}H_4$]-5-CH_3-H_4folate. This observation is in good agreement with the loss of the glutamate group in collision induced dissociation (CID), which generated for labellings in the glutamate and the 4-aminobenzoic acid moiety different product ions. In accordance with these considerations, unlabelled 5-CH_3-H_4folate shows the same abundant product ion as the tracer [$^{13}C_5$]-5-CH_3-H_4folate, since the label of the latter is lost during CID. However, both can be differentiated by the mass increment of the precursor ions.

These results were verified by recording response mixtures of [$^{13}C_5$]-labelled and unlabelled folates with the respective [$^{2}H_4$]-labelled folates. All response curves were linear (R^2 = 0.999 for all curves) for molar ratios of deuterated standards to analytes ranging at least between 0.3 – 4, mostly between 0.2 -20, and could be described by the following response equation:

$$A(IS):A(Analyte) = R_f * n(IS):n(Analyte) + b.$$

Values for R_f and b are shown in Tables 2 and 3. Response curve linearity and good agreement of response factors for [$^{13}C_5$]-labelled and unlabelled folate confirmed the absence of any “cross-talk” effects in the applied mass spectrometer.

Plasma analysis

In our recent report on folate analysis in plasma, erythrocytes and urine [17], we described method improvements such as increased folate stability in MES buffer and improved cleanup on SAX columns. However, in routine analysis of plasma samples, further problems pertaining to folate stability and lifetime of HPLC columns occurred. To increase folate stability, we added 2-mercapto ethanol also to the elution buffer of SAX cleanup.

HPLC column lifetime was improved by precipitation of plasma protein during the extraction procedure. Preliminary studies on folate stability in methanol revealed that over a period of 14 days H_4folate was not stable at -18 °C in a solution containing 50% methanol. However, folate stability at -18 °C was better in solutions with methanol concentrations of 10% and 25% after 14 days. Folates were most stabile in the solution containing 25% methanol, even for the most labile vitamer, H_4folate. For instance, recovery after 14 days of storage at -18 °C was 104% for the latter. Thus all modified extraction buffers contained 12% acetonitrile and 25% methanol.

5-CH_3-H_4folate, the main plasma folate derivative, and 10-HCO-PteGlu (IS) were not detectable in samples analysed including precipitation reagents containing formic acid (Table 2, reagents R1, R2, R3). 5-CH_3-H_4folate peak areas for analysis including R4 and R6 (area counts 16000 arbitrary units) were comparable to the blank (area counts 15000 arbitrary units), for analysis including R5 and R7 5-CH3-H_4folate peak areas were increased by one third (area counts 20000 arbitrary units). The same trend was observed for the IS, however a double-peak was visible for 10-HCO-PteGlu when protein precipitation was accomplished by R7. Thus this approach had to be excluded from the possible precipitation techniques due to the remaining risk that other folate derivatives could be degraded as well. It was concluded that protein precipitation was achieved most efficently by 100% methanol. No adverse effect on plasma H_4folate was observed as the sample was kept in methanol only for a few minutes. Additionally, plasma may be a more protective matrix than the buffers used during the stability experiment.

The sensitivity of the new equipment was further increased by using 1% acetic acid in the mobile phase according to previous reports [20], which resulted in LODs for the single folate vitamers in plasma ranging from 0.25 to 0.62 nmol/L (see Table 3). In comparison with our preceding method [17] the sensitivity was in the same order of magnitude, although a different triple quadrupole mass spectrometer was applied. The recoveries in the present study were determined in blank recombinates after spiking with unlabelled and [$^{13}C_5$]-labelled folates and analysis as detailed before. The obtained recoveries ranged from 94 to 116% and confirmed the method's accuracy. By using [$^{2}H_4$]-labelled folates as the internal standards, losses were almost completely compensated for. Absolute recoveries from SPE clean-up ranged from 32 to 88 % as detailed previously [17].

Analysis of food and ileostomal effluent

Our SIDAs for folates in foods underwent several steps of development in the past and in the present study we report further improvements. In principle, the way of analysis includes extraction in MES buffer after addition of deuterated analogues as IS, followed by incubation with amylase and protease and subsequent deconjugation with rat serum and chicken pancreas conjugase. Cleanup is achieved by SAX and final determination is accomplished by LC-MS/MS.

In this study, we added the quantitation of ^{13}C-labelled monoglutamates to that of the unlabelled folates and increased folate stability by addition of 2-mercaptoethanol to the SAX elution buffer. Moreover, we improved assay sensitivity by reducing the elution volume during SPE sample cleanup from 2.0 mL to 1.5 mL, which led to increased peak signals in mean by 25%. A further experiment by fractionated elution of 1 + 1 mL revealed that endogenous folates were eluted predominantly with the first mL, whereas PteGlu appeared in higher concentrations in the second mL of the elution volume. When comparing fractionated elution and elution with 1.5 mL, the latter was the reasonable compromise in terms of sensitivity and work load. The ratios of unlabelled or [$^{13}C_5$]-labelled folates to deuterated standards, however, did not differ between all elution patterns indicating that no discrimination between the isotopologues occurs as had been reported during affinity chromatography earlier [6].

Ileostomy samples were treated analogously, but had to be lyophilized prior to extraction due to their high water content.

The sensitivity of the assay was demonstrated by LODs ranging between 0.11 to 0.73 µg/100g for unlabelled and [$^{13}C_5$]-labelled folates (Table 4).

The recoveries (n=3) in surrogate matrix starch for unlabelled and [$^{13}C_5$]-labelled folates ranged from 92% to 130%.

Precision of the new assays for folates

Precision of real sample analysis was evaluated in an intra-assay study of a sample prepared several times within one day and in an inter-assay study of a sample prepared on several days within four weeks.

In the intra-assay and inter-assay studies the CVs in plasma ranged from 4 to 15 % and from 4 to 16 %, respectively (Table 3). In foods and ileostomy samples, the CVs in the intra-assay and inter-assay studies ranged from 1 to 6 % and from 8 to 27%, respectively (Table 4)

Application of stable isotope dilution assay to clinical samples

The new SIDAs' suitability was tested by quantifying [$^{13}C_5$]-labelled and unlabelled folates in clinical samples from one individual and in a bread fortified with [$^{13}C_5$]-5-CH_3-H_4folate. The LC-MS/MS chromatograms of the bread analysis (n=4) are shown in Fig. 3. We found the bread to contain 1.2 ± 0.6, 10.2 ± 4.5, 8.1 ± 1.2, 770.9 ± 109.7, 10.7 ± 1.9, 0.7 ± 0.1, 21.4 ± 1.9, 2.5 ± 0.4, 2.1 ± 0.5, and 0.8 ± 0.3 µg/100 g of H_4folate, [$^{13}C_5$]-H_4folate, 5-CH_3-H_4folate, [$^{13}C_5$]-5-CH_3-H_4folate, 10-HCO-PteGlu, [$^{13}C_5$]-10-HCO-PteGlu, 5-HCO-H_4folate, [$^{13}C_5$]-5-HCO-H_4folate, PteGlu, and [$^{13}C_5$]-PteGlu, respectively. The detection of several [$^{13}C_5$]-labelled folates indicated a conversion of [$^{13}C_5$]-5-CH_3-H_4folate during bread making. 27.3, and 10.3 nmol/L of 5-CH_3-H_4folate and [$^{13}C_5$]-5-CH_3-H_4folate, respectively, were quantified in a volunteer's post dose plasma sample after consumption of the test bread. In a post dose ileostomal effluent sample were found 5.42 ± 0.93, 3.37± 0.06, 2.93 ± 0.09, 6.54 ± 0.17, 1.32 ± 0.07, and 0.18 ± 0.03 µg/100g of H_4folate, 5-CH_3-H_4folate, [$^{13}C_5$]-5-CH_3-

H_4folate, 10-HCO-PteGlu, 5-HCO-H_4folate, [$^{13}C_5$]-5-HCO-H_4folate, and PteGlu, respectively.

CONCLUSIONS

The SIDAs presented here are the first reported methods to quantify [$^{13}C_5$]-labelled and unlabelled isotopologues of the most important folyl monoglutamates in plasma, foods, and ileostomal effluent samples.

This new methodology offers the perspective to open up new prospects on folate retention during food processing and human folate metabolism. In foods, the reactivity of single folates and the development of reaction products can be studied by using [$^{13}C_5$]-labelled folates regardless of the endogenous folate spectrum. Additionally, the monitoring of known degradation products or interconversion products such as 10-HCO-PteGlu or 4-aminobenzoylglutamate or identification of new products arising from processing or storage will be possible.

Moreover, ileostomy applications will allow deriving novel information on folate turnover during transition through the gastrointestinal tract. Absorption can be followed specifically using a tracer folate. By quantitation of [$^{13}C_5$]-5-CH_3-H_4folate, bile secretion and reabsorption of dosed folates is observable in the ileostomal effluent. Moreover, the detection of the trace label in plasma enables to assess absorption and distribution of folates and will allow insights in liver metabolism.

As our method is also validated for plasma samples, new inroads into folate bioavailability and metabolism research are accessible. In that way, the combination of an isotopic labelling technique and an AUC/ileostomy model offers the perspective to open new insights in the “black box” liver in folate metabolism.

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Table 1: Investigated folate derivatives in clinical and food samples and MS/MS transitions for their detection

Vitamer	Precursor ion (MH^+) [*m/z*]	Product ion (MH^+ - γ-glutamate) [*m/z*]
H_4folate	446.1	299.2
[$^2H_4$]-H_4folate	450.1	303.2
5-CH_3-H_4folate	460.0	313.2
[$^2H_4$]-5-CH_3-H_4folate	464.0	317.2
[$^{13}C_5$]-5-CH_3-H_4folate	465.0	313.2
10-HCO-PteGlu	470.1	295.2
[$^2H_4$]-10-HCO-PteGlu	474.1	299.2
5-HCO-H_4folate	474.1	327.2
[$^2H_4$]-5-HCO-H_4folate	478.1	331.2
[$^{13}C_5$]-5-HCO-H_4folate	479.1	327.2
PteGlu	442.1	295.2
[$^2H_4$]-PteGlu	446.1	299.2
[$^{13}C_5$]-PteGlu	447.1	295.2

Table 2: Reagents added to plasma for protein precipitation

Reagent number	Final composition in plasma	Additional step [a]
R1	0.1% formic acid in acetonitrile	+
R2	0.1 % formic acid in methanol	+
R3 (modified extraction buffer I)	25% R2, 12% R1, 0.02 mol/L MES, 2% ascorbic acid, 0.1% MCE	-
R4	100% acetonitrile	+
R5	100% methanol	+
R6 (modified extraction buffer II)	25% methanol, 12% acetonitrile, 0.02 mol/L MES, 2% ascorbic acid, 0.1% MCE	-
R7 (modified elution buffer)	40% methanol, 10% acetonitrile, 5% sodium chloride, 1% ascorbic acid, 0.1% MCE, 0.1 mol/L sodium acetate*3H_2O	-

[a] +: additional step to a procedure without protein precipitation -: no additional step

MES 2-(N-morpholino)ethanesulfonic acid; MCE β-mercaptoethanol

Table 3. Validation data for the new SIDAS for plasma folates

Compound	Linearity range of response for the ratio A(IS):A(Analyte)	Parameters for response equation : n(IS):n(Analyte) = R_f * A(IS):A(Analyte) + b		LOD [nmol/ L] (n=3)	LOQ [nmol/ L] (n=3)	Recovery [%] (n=3)	Precision [%] (n=3)	
		slope R_f	intercept b				Intra assay	Inter assay
H_4folate	0.02 – 5.30	0.370	+0.042	0.25	0.51	116±6	6	
5-CH_3-H_4folate	0.02 – 4.60	1.231	-0.033	0.62	1.24	113±8	4	4
[$^{13}C_5$]-5-CH_3-H_4folate	0.03 – 7.80	1.324	-0.014	0.28	0.55	103±1	3	8
10-HCO-PteGlu								
5-HCO-H_4folate	0.15 – 45.45	0.826	-0.489					
[$^{13}C_5$]-5-HCO-H_4folate	0.14 – 42.20	0.826	-0.489					
PteGlu	0.01 – 2.00	0.287	-0.013	0.39	0.77	94±11	15	16
[$^{13}C_5$]-PteGlu	0.01 – 3.60	0.283	0.000					

Table 4. Validation data for the new SIDAS for ileostomal effluents and foods

Compound	Linearity range of response for the ratio A(IS):A(Analyte)	Parameters for response equation : n(IS):n(Analyte) = R_f * A(IS):A(Analyte) + b		LOD [ppb] (n=3)	LOQ [ppb] (n=3)	Recovery [%] (n=3)	Precision [%] (n=3)	
		R**f**	b				Intra assay	Inter assay
H_4folate	0.03 – 4.75	0.341	+0.011	1.98	3.97	99±10	5	14
5-CH_3-H_4folate	0.02 4.50	1.201	+0.018	1.34	2.69	123±8	6	16
[$^{13}C_5$]-5-CH_3-H_4folate	0.04 – 8.35	1.414	+0.005	1.08	2.17	98±8	1	15
10-HCO-PteGlu	0.01 – 1.35	0.156	+0.001	1.06	2.12	101±32	3	16
5-HCO-H_4folate	0.03 – 5.35	0.580	+0.038	2.67	5.34	135±3	4	8
[$^{13}C_5$]-5-HCO-H_4folate	0.04 – 6.70	0.580	+0.038	5.79	11.58	82±5	2	27
PteGlu	0.01 – 6.50	0.388	+0.017	1.98	3.95	130±22	3	16
[$^{13}C_5$]-PteGlu	0.01 – 5.90	0.408	0.000	7.28	14.55	100±17	3	17